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Abstract

Plasma cells develop from B-lymphocytes following stimulation by antigen and express a genetic program aimed at the synthesis of immunoglobulins. This program includes the induction of genes coding for transcription factors such as *PRDM1* and *XBP1*, cell-surface molecules such as *CD138/syndecan-1* and for the unfolded protein response (UPR). We review how the microarray technology has recently contributed to the understanding of the biology of this rare but essential cell population and its transformation into pre-malignant and malignant plasma cells.

1. Introduction

Plasma cells (PCs) – or antibody-secreting cells (ASC) – are the terminally differentiated B lymphocytes devoted to the task of synthesizing immunoglobulins (Ig). Most PCs are located in the bone marrow where they constitute a very small cell population (0.5 % of bone marrow cells) that may survive for months or even years (1). They are the major source of circulating Ig. Antibodies are the effectors of humoral specific immunity, conjugating remarkable specificity and high efficacy in destroying their targets. Because Ig are crucial for the protection of the organism against microorganisms but may be the cause of serious hazard in the form of autoimmunity, the differentiation, proliferation and survival of PCs are tightly regulated. The high specificity of the antibodies is the result of several DNA modifying events involving mutations in variable Ig gene mutations and Ig chain switch. Error in Ig switch process can lead to an illegitimate activation of oncogenes that are considered as the first step of a process that changes a B lymphocyte into an uncontrolled proliferating premalignant or malignant PC in the bone marrow of patients with monoclonal gammopathy with undetermined significance (MGUS) or with multiple myeloma (MM). We will review how microarrays have recently contributed to the understanding of the biology of this rare but essential cell population and its transformation into pre-malignant and malignant PCs.

2. Normal plasma cells

Using gene expression profiling (GEP) with Affymetrix microarrays on purified cell populations of plasmablasts and bone marrow mature PCs, we were recently able to build a molecular portrait of 125 genes involved in differentiation of B cells into plasmablasts and subsequently into bone marrow PCs (BMPCs) (2). We are currently extending these results by comparing the molecular profiling of 7 normal bone marrow BMPC samples and 7

plasmablasts samples, to that of 7 B memory lymphocytes samples using pan genomic microarrays. This genome wide screen shows that 2482 probes (5.4% of the 44 760 probes used) are differentially expressed between memory B cells and BMPC with a p-value ≤ 0.01 (Kruskal-Wallis non parametric test) and a fold change of a least 3 (manuscript in preparation). These modifications include transcription factors, cell surface molecules and enzymes involved in metabolism, especially in protein processing.

2.1. The plasma cell transcription factor network

Differentiation of a B lymphocyte into a PC is driven by a profound modification of the transcription factor network (Figure 1A). This has been comprehensively reviewed by K. Calame and collaborator (Shapiro-Shelef and Calame 2005). The transcriptional repressor *PRDM1/PRDI-BF1/BLIMP1* is the master regulator of terminal B cell development and is sufficient to drive primary mouse splenocytes into antibody-secreting PCs (3). Lack of *PRDM1* in B lymphocytes causes a profound defect in Ig synthesis due to a lack of PC formation (4). *PRDM1* expression is increased over 4 times during PC differentiation, as early as in proliferating plasmablast stage (Figure 1B). Genes regulated by *PRDM1* have been extensively studied by microarrays (5). Genes identified as directly repressed by *PRDM1* due to binding sites to this transcription factor in their promoter include *PAX5, SPIB/Spi-B, ID3, CTIIA* and *MYC* (5, 6). *PRDM1* expression also blocks immunoglobulin class switching by blocking the expression of *AID, KU70, KU86, DNA-PKCs* and *STAT6* (5). One key regulator of *PRDM1* expression was identified by a microarray screen, the six-zinc-finger-containing transcriptional repressor *BCL6* (7). *BCL6* is essential for the generation of germinal centers where it is highly expressed and prevents the expression of the *TP53/p53* suppressor gene (8) and the expression of *PRDM1* (7). Repression by *BCL6* depends on *MTA3* expression, a subunit of the corepressor complex *MI-2/NuRD* (9). Downregulation of *paired box protein 5*

(PAX5)/BSAP gene is one of the main consequence of the upregulation of *PRDM1* and is necessary for the PC differentiation program to proceed (10). *PAX5* is essential for B-lineage commitment and activates the expression of genes such as *VpreB* and *CD19* and represses the expression of genes switched on in PCs such as *heavy chain Ig* (*IgH*) and *XBP1*. Other transcription factors that are characteristic of B lymphocytes are silenced upon plasma-cell differentiation, such as *BACH1* and *BACH2* (11), or *EBF* (12). The modifications of this transcription factor network are comprehensively captured by microarrays as shown in Figure 1B. Other transcription factors important for PC formation include *IRF4* and *OBF1* – the later only for T-cell dependant PCs (13, 14). Our results show that *IRF4* is highly expressed at the plasmablast stage whereas OBF1 only show a modest increase during PC differentiation.

Most interestingly, the microarrays also uncover new transcription factors whose expression is strongly induced during PC differentiation. One of these transcription factors is *BHLHB3/DEC2/SHARP1* (unpublished results), a class B basic helix-loop-helix transcription factor involved in the regulation of the molecular clock of the body (15). This transcription factor displays a remarkable high expression in normal PCs (Figure 1B) that is also observed in MM cells (data not shown). This pattern of expression strongly suggests that *BHLHB3* may play a functional role during late B cell differentiation.

2.2. The plasma cell phenotype

Based on studies of reactive plasmacytosis and in vitro culture systems, at least three different PC stages can be identified by cell surface phenotyping during the differentiation process that transforms a germinal center B lymphocyte with a high affinity Ig to a mature PC residing in the bone marrow. The plasmablast is a $CD20^{-}$ $CD38^{++}$ $CD45^{++}$ $CD126^{++}$ $CD138^{-}$ cell located in secondary lymphoid organs and transiently in the blood stream, and can be obtained from resting B memory cells in vitro (16-18). In the bone marrow, two populations differing in

their respective *CD45* expression are found, early PCs that are $CD20^{-}$ $CD38^{++}$ $CD45^{++}$ $CD126^{++}$ $CD138^{++}$ whereas mature PCs are $CD20^{-}$ $CD38^{++}$ $CD45^{weak}$ $CD126^{+}$ $CD138^{++}$. As illustrated by microarrays experiments (Figure 2), PC differentiation is characterized by the loss of pan-B markers (*CD20, CD22, CD24*), the up-regulation of *CD38* starting from the plasmablast stage and the appearance of *syndecan 1/CD138* late during development. In agreement with the role of IL-6 during PC differentiation, plasmablasts and/or mature PCs express 5 times more *IL-6R/CD126* and *GP130/CD130* than memory B cells (Figure 2). Down-regulation of *CD45* expression characterizes the terminally differentiated, non proliferating mature PC stage. This characteristic is shared by primary myeloma cells (19). Conversely, a high expression of *CD45* is observed on plasmablasts and early PCs as well as the proliferating fraction of the U266 human myeloma cell line (HMCL) (17, 20). Other surface markers vary during PC differentiation, including *CD31* and *CD54*, which are found on mature PCs (16), *CD39* which is specifically found on plasmablasts (Figure 2, unpublished results) and *VCAM1*. Some of these cell surface molecules (*CD20, CD38, CD138*) have been used to highly purify plasmablasts, tonsil or bone marrow PC by flow cytometry or using magnetic beads, and thus allowing a more specific molecular analysis using microarrays (2, 16, 21, 22).

2.3. The plasma cell homing

PC differentiation is a dynamic process during which a B lymphocyte expressing a high affinity Ig will leave the germinal center of secondary lymphoid organs to migrate, via the blood, to the bone marrow which is the principal site of long-lived ASC. The mature PC depends on survival signals provided by the microenvironment niche of the bone marrow. These “survival niches”, in which long lived PCs reside, appear to be limited in number. There is presumably competition among PC for these niches. Evidence for this hypothesis is

given by the fact that long lived PC without tetanus toxoid specificity are expulsed in the blood circulation after vaccination with tetanus toxin (23). The movement of the PC is under the control of repellent and attractant signals, involving chemokines, chemokine receptors, and adhesion molecules (24).

2.3.1. Chemokines

The chemokine receptor *CXCR4* is a major determinant of the medullar localization of immature B lymphocytes and ASC. B-cell lineage and granulocytic precursors are released into the periphery in *CXCR4*$^{-/-}$ mice (25). In addition, reconstitution of hematopoiesis in irradiated mice with *CXCR4*$^{-/-}$ fetal liver cells results in the aberrant shift of PCs from the bone marrow to the blood circulation (26). Accordingly, *CXCR4* expression is downregulated in plasmablasts which transit through the blood flow, before regaining a strong expression in BMPC (Figure 3). The mobilization of ASC is also linked to the loss of expression of two chemokine receptors, *CCR7*, a receptor for *CCL19/ELC* and *CCL21/SLC*, and *CXCR5*, a receptor for *CXCL13/BLC* (26). Both chemokine receptors are involved in the localization of B lymphocytes in secondary lymphoid organs, i.e. in the T-cell zone and the germinal centers, respectively (27). Loss of *CCR7* and *CXCR5* will increase the differentiating ASCs to respond to *CXCL12/SDF1*, the ligand of CXC*R*4, that is highly expressed by bone marrow stromal cells.

We previously observed, using cDNA macroarrays, that malignant PCs overexpress the chemokine receptors *CCR1* and *CCR2* as compared to lymphoblastoid cell lines (28). Microarray analysis of primary plasma cell samples has shown that *CCR1* and *CCR2* are overexpressed in both normal and malignant PC compared to B memory lymphocytes, *CCR2* is expressed starting at the plasmablast stage whereas *CCR1* is only overexpressed in mature PC stage (Figure 3) (2, 16). The expression of *MIP1alpha*, one of the ligands of *CCR1*, is

induced during PC differentiation (see below, Figure 5). The preferential expression of these chemokine receptors is likely governing the homing of BMPCs to their bone marrow niche.

2.3.2. Selectins and adhesion molecules

Plasmablasts transit from secondary lymphoid organs to the bone marrow. The mechanisms by which ASCs traffic are still unsatisfactorily understood, but adhesion and activation events regulating the migration of all subsets of circulating leukocytes are likely to apply to ASCs too (24). Circulating leukocytes tether to and roll on the endothelium through transient selectin–carbohydrate ligand interactions and/or integrin–CAM (cell cell-adhesion molecule) interactions. Rolling allow the cell to become sufficiently activated by locally released or displayed cytokines and chemoattractants to allow an integrin-mediated firm adhesion followed by diapedesis. By providing a large scale gene expression analysis of plasmablasts, microarrays have identified *SELPLG/CD162/Selectin-P ligand* as being strikingly upregulated during in vitro plasmablast generation (Figure 4) (16). In turn, the *selectin-L/SELL/CD62L* is lost between the stages of plasmablast and BMPC.

Regarding integrins, figure 4 shows that two alpha chains are preferentially expressed in ASCs (2, 16). *ITGA6* expression is switched on in plasmablasts and still increases when reaching the stage of BMPC as shown previously (29). *ITGA8* is only expressed in BMPC. Because the integrin beta chain *ITGB1* is expressed by PCs (Figure 4), the corresponding functional integrin heterodimers (α6β1 (VLA-6) and α8β1 (VLA-8)) are likely expressed on these cells. Conversely, the *integrin alpha M/ITGAM/CD11b* is strikingly downregulated early during PC differentiation. A very similar expression pattern of integrin alpha chains is found on malignant PCs (data not shown).

Other adhesion molecules upregulated during PC differentiation include *vascular cell adhesion molecule 1* (*VCAM1*), *intercellular adhesion molecule 2 (ICAM2)* and the tetraspanin *CD9* (Figure 4) (2).

2.4. Integrating extracellular cues

Interleukin-6 (IL-6) plays an important role in the normal PC physiology. Mice lacking this gene are impaired in T-cell-dependent antibody response (30), and conversely, transgenic mice expressing IL-6 under the control of the *Eμ* enhancer promoter trigger a fatal polyclonal plasmacytosis (31). In agreement with these findings in mice, *IL-6* is a growth factor for nonmalignant human plasmablasts (32). As highlighted above, both chains of the *IL-6* receptor are upregulated during PC differentiation (Figure 2), enabling the PC to respond to *IL-6*.

Most long survival PCs home to the bone marrow. A privileged relationship between PCs, bone and the cells that mediate bone homeostasis, *i.e.* osteoblasts and osteoclasts, is very likely, but still very poorly characterized. By contrast, it is well documented that during the development of multiple myeloma, malignant PCs activate a large number of cell-to-cell signals that lead to the blockade of osteoblast activity and the concomitant stimulation of osteoclast activity (33). Whether this program is specific to the transformed PC or, at least in part, the execution of a physiological program that allows the nonmalignant PC to carve a survival niche in the bone marrow is an issue that awaits to be carefully addressed. It is therefore interesting to observe that microarray analysis of PCs identifies the secreted *WNT* antagonist *FRZB/sFRP3* as a gene induced in BMPC (Figure 5) (28). Indeed, the *WNT* signaling pathway is important for the growth and differentiation of osteoblasts. This is illustrated by osteopenia in mice with loss of function of the *WNT* co-receptor *LRP5* or in mice exposed to elevated levels of the *WNT* inhibitor *DKK1* (34). It must be noted that the level of expression of *FRZB/sFRP3* in BMPC, though significantly elevated as compared to memory B cells and plasmablasts, is way below the level found in most MM samples (Figure

5). We have also observed that the chemokine *MIP1alpha/MIP1A/CCL3* is induced during PC differentiation. This chemokine has been shown to be an osteoclastogenic factor (35).

Microarray analysis of human PCs has revealed the preferential expression of B cell activating factor *BCMA/TNFRSF17* on plasmablasts and mature PCs, revealing *BCMA* as a typical PC gene (Figure 5) (2, 16, 36). *Transmembrane activator and calcium signal–modulating cyclophilin ligand interactor (TACI)/TNFRSF13B* is a closely related receptor that is also induced during PC differentiation (Figure 5). *BCMA* and *TACI* are members of the *TNF receptor family* with two ligands of the TNF family: *A Proliferation-Inducing Ligand APRIL/TNFSF13 and B Cell Activating Factor BAFF/TNFSF13B*. In addition, *BAFF* also ligates a third *TNFRSF* receptor, *BAFF-Receptor BAFFR/TNFRSF13C*. Studies with knock-out and transgenic mice has shown that the *BAFF/BAFFR* axis has an essential role in the maturation and the survival of peripheral B cells, *BCMA* has a critical role for the survival of long-lived bone marrow PCs, whereas *TACI* participates in T cell–independent immune responses and isotype switching (37-40). The expression of *APRIL* in B cells and mature plasma cells (Figure 5) and the very strong expression of *APRIL* in osteoclasts, monocytes and the myeloid lineage (41) suggests that plasma cells are exposed to the cognate ligand of *BCMA* in their bone marrow niche. Interestingly, it was recently established that *APRIL* can bind syndecan-1, a proteoglycan that is a hallmark of PC differentiation (42). These findings strongly suggest that *BCMA* plays an important role in human PC survival as it does in mice, with consequences for therapeutic intervention in non-malignant or malignant plasma cell disorders like MM (discussed below).

PCs are the main hematopoietic cells that express the heparan sulfate proteoglycan *syndecan-1/SDC1/CD138* (43). In addition to PCs, preB lymphocytes may also express CD138, but not mature B lymphocytes, nor the other hematopoietic cell populations (44). This unique feature has opened the way to identify and to purify normal and malignant PCs

from bone marrow or blood to homogeneity using magnetic bead selection (22, 45). *SDC1* is a typical late PC gene, whose expression begins after the plasmablast stage (Figure 2). Interestingly, in addition to a role in cell adhesion by promoting homotypic cell aggregation, a crucial function for *SDC1* is to be cofactor in many signalling pathways by binding heparin binding growth factors, thereby increasing the availability of these molecules to the PCs (46). The list of secreted soluble ligands sequestered by *SDC1* is rising and includes *Hepatocyte Growth Factor (HGF), Osteoprotegerin (OPG), Fibroblast Growth Factors (FGFs),* chemokines, some members of the TGF superfamily, and very recently *APRIL* (42, 46, 47).

Four years ago, we found a major expression of the tyrosine kinase *TYRO3* in some HMCL. TYRO3 is part of a family of tyrosine kinase transmembrane receptors that also encompasses the closely related receptors *MERTK* and *AXL*. This family of tyrosine kinase is involved in several physiological processes, including amplification of platelet aggregation, control of inappropriate immune responses, spermatogenesis and regulation of osteoclastic bone resorption (48-51). In addition, these receptors are overexpressed by many tumors and can transform cultured cells in vitro (52, 53). We gathered a remarkable body of expression data regarding this signalling pathway in plasma cells. Although *TYRO3* is not expressed in normal PCs or primary malignant PCs, *MERTK* and *AXL* are expressed at the terminal stage of PC differentiation as shown in Figure 5. Moreover, the expression of the ligand of these three receptors, *Growth Arrest-Specific 6* (*GAS6*), is turned on in PCs. These observations give compelling evidence that this signalling pathway is involved in the biology of ASC. *TYRO3* activation by *GAS6* induces osteoclastic bone resorption in mice (49), thus this signaling axis could play a role in the physiological interaction of PCs with bone. However, this hypothesis does not clarify the meaning of the upregulation of *TYRO3* in some HMCL. Therefore, careful functional analysis will be mandatory to determine the exact role of the expression of these receptors and their ligand by normal and malignant PC.

2.5. Apoptosis

Memory B cells and BMPC are cells with a prolonged survival in vivo, in contrast to plasmablasts that are cell highly prone to apoptosis and die if they are deprived of the access to a survival niche. Expression of several anti-apoptotic members of the *BCL2* family varies accordingly. Memory B cells express high levels of *BCL2A1/A1 and BCL2* (Figure 6). BMPC express markedly *BCL2 and MCL1*. In contrast, plasmablasts express no or little of these anti-apoptotic molecules. These characteristics may explain why plasmablasts are doomed to die by apoptosis as observed for plasmablasts after 8 days of in vitro differentiation of peripheral blood B lymphocytes or for plasmablasts from reactive plasmacytosis (16, 32). It is likely that in the case plasmablasts reach the appropriate bone marrow niche, they receive signals that result in the expression of antiapoptotic proteins such as *BCL2 and MCL1*. Another observation in line with the pre-apoptotic state of plasmablasts is the expression of *caspase 6* (*CASP6*) by these cells (2).

2.6. Cell cycle

Peripheral blood memory B cells and BMPC are cells that are not cycling, in contrast to plasmablasts that can have more than 30% of the cells in the S-phase (16). It is therefore not surprising to observe in plasmablasts the very strong expression of more than one hundred genes involved in the cell cycle progression (unpublished results). Some typical examples are depicted in Figure 6, such as the Budding Uninhibited by *Benzimidazoles 1 homolog (BUB1)* gene or the *minichromosome gene MCM2*. Of note, *cyclin D2 (CCND2)* is the cyclin D that is highly expressed in proliferating non-malignant plasmablasts, whereas *CCND1* is not expressed and *CCND3* expression level does not vary markedly during PC differentiation.

2.7. Immunoglobulin secretion and the unfolded-protein response

During PC differentiation, as a consequence of the induction of *PRDM1* and the repression of *PAX5*, Ig production is switched to a secreted form and is massively upregulated. This results in the overflow of the polypeptide processing and folding machinery in the endoplasmic reticulum (ER) and generates a stress signal to the cell. This warning is the primary signal for a highly coordinated response called the unfolded protein response (UPR) resulting in the production of ER chaperones and enzymes assisting in the protein folding. Thus, the UPR adapts the capacity of the secretory system to the load of the protein synthesis by transcriptional up-regulation of genes that function in all aspects of ER protein processing and metabolism. The link between plasma cell differentiation and UPR was demonstrated by the finding that the *X-box binding-protein 1 (XBP1)* basic-region leucine zipper transcription factor, which was found as the functional equivalent to the yeast *Hac1p* UPR activating transcription factor (54), was also shown to be required for PC differentiation (55). Microarray technology has significantly contributed to the understanding of the complex but coordinated UPR in mammalian cells. The molecular profiling induced by the expression of *XBP1* was studied in mice in wild type and *XBP*1-/- B-cells and embryonic fibroblasts, and in the human RAJI lymphoma cell line. Major proteins involved in this coordinated UPR are schemed in Figure 7A.

UPR is turned on in mammalian cells by the activation of three bZIP transcription factors: *ATF6, ATF4 and XBP1*. Following ER stress, the membrane-tethered precursor of *ATF6* is relieved from its interaction with the *Heat Shock 70kDa Protein 5*/*HSPA5*/*BIP*, is cleaved and the N-terminal DNA-binding domain is translocated to the nucleus where it activates the transcription of *XBP1* (Figure 7A). In parallel, the transmembrane kinase and endonuclease *Endoplasmic Reticulum to Nucleus Signalling 1 ERN1*/*IRE1* is also liberated from *HSPA5*, resulting in the unconventional splicing of XBP1 which thereby becomes fully functional. The transcriptional activity of *ATF6* and *XBP1* are the corner stone of the

expression of the UPR. In PCs, *XBP1* expression is upregulated and spliced (56), and accordingly, many genes induced during PC differentiation are also found induced by the overexpression of *XBP1* alone (57, 58). This common profile includes genes targeting proteins across the ER membrane (*SRP54*, *RPN1*, etc.), translocation of proteins across the ER membrane (*SSR1*, *SSR3*, *SSR4, SEC61A1*, etc.), folding of ER proteins (*DNAJB9*/*ERdj4*, *HSPA5, DNAJC3, DNAJB11*/*HEDJ* etc.), oxidative protein folding, i.e. formation of disulfide bonds (*ERO1L*), the degradation of misfolded protein (*EDEM2, SEL1L, HERP*/*HERPUD1*, etc.), protein glycosylation (*SLC33A1, DDOST, FUT8*, etc.), ER-Golgi trafficking (*SEC23B*, *ARCN1*, etc.). Interestingly, the third bZIP transcription factor, *ATF4* does not seem to be active because two major *ATF4* downstream targets, *CHOP* and *GADD34*, have expression level similar in BMPC and B memory cells. Activation of *CHOP* is part of an ER stress program that results in protein translation stop and apoptosis (59). Absence of significant activation of this pathway suggests that the physiological load of newly synthetized Ig is correctly handled by the UPR and that the stress response resulting in *CHOP* activation is not triggered during normal PC differentiation (57). Our current data shows the power of microarrays to investigate as a whole the expression of UPR genes in PC differentiation. Figure 7B shows 22 UPR genes that are upregulated in plasmablasts and PC compared to memory B cells.

3. Input of microarray for the understanding of malignant plasma cell dyscrasias

3.1. Short outline of malignant plasma cell dyscrasias and gene abnormalities

3.1.1. Malignant plasma cell dyscrasias.

MM is a B-cell neoplasia that affects about 15 000 new patients per year in Europe and about the same number in the United States. The median age at diagnosis is 67 years. MM is characterized by the accumulation of a clone of plasma cells in the bone marrow. In a

minority of patients with plasma cell leukemia (PCL), myeloma cells can grow and survive without the support of the bone marrow environment. The MM clone harbors somatic mutations of the immunoglobulin genes, which remain stable throughout the disease (60). This indicates that the "myeloma stem cell" originates from a cell that underwent antigen selection in the germinal centers, either a memory B cell or a plasma cell. High dose chemotherapy (HDT) increases the median overall survival of newly diagnosed patients to about 5-7 years in randomized clinical trials, though this is still a matter of discussion (61). The advent of new compounds such as thalidomide (62), its derivatives or bortezomib (63) has improved the treatment of patients in relapse from high dose chemotherapy and may also improve induction treatment before HDT (64). Compared with the general population, MM occurs more frequently in patients with MGUS, a pre-malignant condition, with an annual rate of transformation of MGUS into MM of about 1%. MGUS is frequent in the elderly, about 3% of the individuals over 70 years old (65).

3.1.2. Chromosome abnormalities in MM.

A major advance in the biology of MM has been the identification of gene abnormalities using conventional cytogenetics or fluorescence in situ hybridization (FISH). This field was recently reviewed by Fonseca et al. (66). Karyotypic instability is a hallmark of MM (67). By metaphase cytogenetics, chromosome abnormalities can be detected in about 30% of patients with newly diagnosed MM. Using FISH on CD138-selected cells or with immunostaining of intracytoplasmic immunoglobulin light chains to identify plasma cells, chromosome aberrations can be detected in virtually all patients at the time of first diagnosis. Based on the data of metaphase cytogenetics, two partially overlapping pathogenetic pathways have been proposed: non-hyperdiploid MM (comprising MM with hypodiploid, pseudodiploid and near-tetraploid karyotypes), characterized by recurrent translocations involving the

immunoglobulin heavy chain gene (IgH) on chromosome 14q32.3 and with a high incidence of 13q loss, and hyperdiploid MM associated with multiple trisomies involving chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, but a lower incidence of IgH-translocations and 13q aberrations (68-70). About 50% of MM patients have hyperdiploid tumors. Recently, interphase FISH-based assays distinguishing between hyperdiploid and non-hyperdiploid MM have been reported (71, 72).

IgH-translocations are found in about 50% of patients with MGUS, 55-70% of patients with intramedullary MM, 80% of patients with primary PCL, and 90% of human myeloma cell lines (HMCL). Translocations affecting the immunoglobulin light chain genes (*IgL*) occur less frequently (< 20%) and mostly involve the *IgL*-λ locus. Chromosome partners have been identified in 40% of the cases and include chromosome 11q13, resulting in the deregulation of *CCND1* (*cyclin D1)* and *MYEOV* in 16% of the patients, chromosome 4p16 resulting in the deregulation of *FGFR3* and *Wolf-Hirschhorn syndrome candidate 1* (*WHSC1/MMSET)* in 15% of MM patients, chromosome 16q22-23 resulting in the deregulation of *c-maf* in 5% of MM patients and chromosome 6p21 resulting in the deregulation of *CCND*3 (*cyclin D3*) in 4% of the patients. *IgH*-translocations define distinct entities of MM with clinical, morphologic, immunophenotypic, and therapeutic implications (66, 73, 74). Especially, the translocation t(4;14)(p16.3;q32.3) confers a bad prognosis even after high-dose therapy and autologous blood stem cell transplantation (75). *IgH*-translocations are also found in patients with MGUS indicating that they are early and possibly seminal events in the premalignant phase of the disease (66, 76).

We have recently described a model for the clonal evolution of MM from initiation to first diagnosis based on results from interphase FISH (72). According to this model, translocation t(11;14), hyperdiploid, and non-hyperdiploid-karyotypes each define a distinct subgroup of the disease. Furthermore, patients with additional copies of chromosome 1q21 could represent

a poor prognosis group, indicated by significantly higher beta-2-microglobulin and lower hemoglobin levels. Deletions of 22q11, 8p12, 6q21 and 17p13 (*TP53*-locus) were found as subsequent or late events occurring in the course of the disease, but not defining subentities of MM.

Deletion of chromosome 13q14 can be found by interphase-FISH in about 50% of patients with newly diagnosed MM. This aberration has repeatedly been described to be associated with poor prognosis. Its significance was confirmed for patients after autologous and after allogenic stem cell transplantation following dose-reduced induction therapy (77, 78). However, the translocation t(4;14) is in almost all cases associated with a deletion of 13q14, and overall survival seems to be significantly shorter for patients with both t(4;14) and deletion of 13q14 than for those with deletion of 13q14 alone (77).

Deletions of chromosome 17p13 can been found in about 10-30% of patients with newly diagnosed MM (72, 79). This aberration has been reported to confer a grave prognosis for patients treated by conventional therapy. Furthermore, deletion of 17p13 was described to be an adverse prognostic factor following autologous stem cell transplantation(77, 80).

RAS mutations are found in 35-50 % of newly diagnosed patients (66). *RAS* mutations are rare in patients with MGUS and increase in patients with progressive disease, suggesting that they contribute to disease progression.

3.2. Gene expression profiling (GEP) in MM and patient classification

3.2.1. GEP and patient classification

Gene expression profiling of myeloma cells is thought to be utilizable to define distinct MM entities with different prognosis, and therefore stratify patients for adapted treatment. A first study comparing GEP of purified myeloma cells obtained from newly diagnosed patients with

MM or MGUS, of HMCLs and of purified bone marrow plasma cells from healthy individuals was published in 2002 by the group of John Shaughnessy (81). Using hierarchical clustering, myeloma cells could be subdivided into four groups. One group comprised the HMCL whereas another group integrated the normal bone plasma cells and MGUS samples. In the "HMCL-like" myeloma cell group, patients tended to have poor-risk profile including a higher serum β2m values and unfavorable cytogenetic abnormalities. Furthermore, the same group used microarrays to define molecular signatures of cells representing the stages of human B cell development, peripheral blood B cells, tonsillar B cells, tonsillar plasma cells and bone marrow plasma cells. They showed that myeloma cells from high-risk MM has a tonsillar B cell signature whereas myeloma cells from patients with low risk feature have bone marrow or tonsillar plasma cell signature (82). Using the expression profiles of 221 MM patients assessed with Affymetrix U95 microarrays, a different classification of patients into four groups with distinct cytogenetic characteristics (ploidy status, *IgH*-translocations, trisomy 11) and clinical parameters (β2m, IgA, albumin, creatinin, MRI lesions, and event free survival (EFS) and overall survival (OS)) was published (83). 100 genes were identified correlating with the EFS. Three genes had independent prognostic value: *Ras-related nuclear protein* (*RAN)*, *Zinc finger and homeodomain protein 2* (*ZHX-2)* and *Chromosome condensation 1-like* (*CHC1L)* (83). Patients with high *RAN* expression and low *ZHX-2* and *CHC1L* expression had a relatively shorter EFS compared to patients with low *RAN* and high *ZHX-2* and *CHC1L* expression. *RAN* encodes for a GTPase involved in several biological pathways: shuttle of protein and RNA through the nuclear pore complex, regulation of chromosome condensation, spindle formation, nuclear assembly and cell cycle progression. *CHC1L* is located on chromosome 13q14.3 and is a homolog of *RCC1* which is a GTP exchange factor of *RAN*. *ZHX-2* is a negative regulator of the *NF-Y transcription factor*,

which is involved in cell cycle control. Accordingly, a negative correlation of expressions of *ZHX-2* and a panel of 30 proliferation genes was found.

Currently, published GEP classifications vary considerably. A careful statistical validation on a sufficiently high number of patients, a longer follow up and testing in a prospective clinical trial is mandatory before a GEP classification might be used for clinical decisions.

3.2.2. GEP and gene abnormalities

The first reports combining GEP and cytogenetic data indicated that the 2 most prevalent translocations in MM are associated with high expression of the deregulated oncogenes (81). In patients with a t(4;14) translocation, a high expression of WHSC1/*MMSET* and *FRGR3* is found in 100% and 80% of cases, respectively (84, 85). The t(11;14) translocation is associated with a high *CCND1* expression. CCND1 is not expressed in normal lymphoid and hematopoietic cells, particularly normal plasmablastic and plasma cells (16) (Figure 6). Using expression profiles of 65 newly-diagnosed MM-patients obtained with Affymetrix U133 A+B microarrays, we confirmed that myeloma cells with t(4;14) and t(11;14) translocations can be easily visualized by a prominent expression of *FGFR3*, WHSC1/MMSET or *CCND* genes (see Figure 8). *CCND1* expression is also found in myeloma cells without t(11;14) translocation (28) (Figure 8), either in the context of a light chain translocation, or correlated with a gain of the chromosomal region 11q13. Using microarrays, Bergsagel et al. point out that a high expression of one of the three cyclin D genes – *CCND1, CCND2 or CCND3* – is a general feature of MM and proposes a classification of patients with MM within 8 groups (86).

Combining FISH and GEP, we identified a 6-gene predictor for four groups of patients: group 1.1 with intermediate *CCND1* expression and gain of 11q13 without a translocation involving 11q13, group 1.2 with high CCND1 expression and a translocation involving 11q13, mostly t(11;14)(q13;q32.3), group 2.1 with CCND2 expression without elevated *FGFR3* expression,

and group 2.2 with *CCCND2* expression, t(4;14) translocation and *FGFR3* expression (87). This 6-gene predictor correctly classifies all patients in a training group of 65 patients, and about 90% of patients of an independent validation group comprising additional 65 patients We also found that the patients predicted to be in group 2.2 had the shortest event-free survival, in agreement with the known bad prognosis of t(4;14) (66). GEP can give some insight into the role of del13q analyzed by FISH or conventional cytogenetic (88) and indicates an upregulation of the ribosomal machinery in hyperdiploid clones with a low incidence of del13q (89). Presently, no data are published regarding the ability of microarrays to predict for del17 (*P53* locus) that is associated with a poor clinical outcome after HDT (77). Thus, the combination of gene expression profiling and molecular cytogenetics like iFISH or aCGH is a promising approach and might facilitate the interpretation of expression data in the future.

3.3. GEP and the tumor microenvironment

3.3.1. Short outline of MM clone and tumor environment

Although MM is characterized by a small growth fraction, the rate of proliferation is one of the best prognostic factors (90). In a majority of patients, the survival of myeloma cells is strongly dependent on interaction with the bone marrow environment (91), including endothelial cells, osteoblasts, osteoclasts, monocytes, and polymorphonuclear leukocytes. Myeloma cells, in turn, influence the bone marrow environment, e.g. by producing VEGF that stimulates neoangiogenesis (92). Angiogenesis is a marker of disease activity (93). Interaction between myeloma cells and bone marrow stromal cells has been widely documented. In particular, myeloma cells can trigger IL-6 production in bone marrow stromal cells through an *NF-□B*-dependent pathway (94, 95). The increased osteoclast activity in MM is probably the result of a direct contact of these cells with myeloma cells (96). The crosstalk between

myeloma cells and the bone marrow environment involves chemokines and chemokine receptors, mainly *CCR1* (97), *CCR2* (98), *MIP*-1α (35) and *MIP-1*β (99), adhesion molecules (100), metalloproteinases (101) and production of growth factors (102). We and others identified *IL-6* as a major myeloma cells growth factor in 1989 (103, 104), mainly produced by the microenvironment, in particular monocytes and stromal cells (105). I*GF-1, hepatocyte growth factor, IL-10, IL-21* and more recently the *epidermal growth factor family (EGF)* and the *BAFF/APRIL* B cell growth factors were identified as myeloma cells growth factors (106-108).

3.3.2. Input of microarrays to identify novel myeloma cells growth factors

DNA arrays have proven to be very useful for identifying novel myeloma cells growth factors. In our first transcriptome study of MM, we compared the GEP of HMCL and EBV transformed cell lines using nylon macroarrays dedicated to the analysis of 268 genes coding for growth factors or their receptors, and we identified several genes whose gene products were demonstrated to be important in myeloma cells biology: the *heparin binding epidermal growth factor like growth factor (HB-EGF),* the *WNT* pathway decoyed receptor (*FRZB/sFRP3*), the *TYRO3* receptor for *GAS6* factor, the *IL-6 receptor,* the *IGF-1 receptor*, the *hepatocyte growth factor activator (HGFAC)* (28). The use of microarrays measuring the expression of more than 30 000 genes has confirmed and extended these early findings.

<u>EGF family in MM.</u>

HB-EGF is expressed and used by some HMCLs as an autocrine growth factor, acting in synergy with *IL-6* (28, 109). Using microarrays, we were able to screen the expression of the EGF family and their receptors and provide a general picture of the involvement of this signalling pathway in MM. The EGF family comprises 10 members and at least 4 of them can bind heparan sulfate chains: *HB-EGF, amphiregulin, neuregulin-1, neuregulin-2* (110). This family binds to and induces the homo- or heterodimerization of three receptors: the

EGF receptor *ErbB1, ErbB3, and ErbB4*. *ErbB2* is a fourth member unable to bind EGF ligands. *ErbB2* is a preferred heterodimerization partner for all other *ErbB* members and increases *ErbB* receptor signaling (111).

First, expression of *EGF* receptor family is a feature of plasma cell differentiation. Normal plasma cells express the *ErbB1* and *ErbB2* receptors unlike normal memory B cells or normal plasmablastic cells (112). Malignant plasma cells also expressed *ErbB1*, *ErbB2* and in addition *ErbB3* and *ErbB4*. Second, we found that *HB-EGF* is not expressed by primary myeloma cells but by the bone marrow environment, mainly by monocytes, polymorphonuclear leukocytes (107). More generally, only the *EGF* family members that are able to bind heparan sulfate chains (*HB-EGF*, *amphiregulin, neuregulin-1, neuregulin-2*) are able to trigger myeloma cells growth. This property is linked to the ability of these *EGF* members to bind at a high density to the surface of myeloma cells, through syndecan-1 (113) (Figure 9). *Syndecan-1* is a heparan sulfate proteoglycan induced in plasma cell differentiation together with some *EGF* receptor members. As illustrated in Figure 9, one of the roles of syndecan-1 is to be a coreceptor for heparan sulfate binding EGF members favoring activation of specific *ErbB* receptor. Of interest, some myeloma cells acquire the capacity to produce *HB-EGF* initially produced by the microenvironment, which may confer an advantage for growing independently of the tumor environment. In parallel to these expression data, it was shown that the activation of *ErbB* receptor acts in synergy with activation of *gp130 IL-6 transducer* in some myeloma cell lines (109) and in epithelial cells (114, 115). In particular, Jelinek and collaborators demonstrated that *ErbB3* activation can trigger phosphorylation of interferon receptor on myeloma cells (116). We found that a pan *erbB* kinase inhibitor can induce apoptosis of primary myeloma cells cultures for several days together with their bone marrow environment, further emphasizing the relevance of *EGF* family members to trigger primary plasma cell survival (107). These findings are of interest

since clinical grade inhibitors of the *EGF* receptor family are now under investigation in epithelial cancers (117).

BAFF/*APRIL* growth factors

Another example for the usefulness of microarrays in investigating the mechanisms of the growth of myeloma cells is provided by studies on *BAFF* and *APRIL*. These growth factors are essential for nonmalignant B lymphocyte and plasma cell development (see above). In an initial comparison of expression profiles of normal plasmablasts, bone marrow plasma cells, and B cells, we found that *TACI* and *BCMA* genes were overexpressed by plasma cells (16). We and others further demonstrated that *BAFF* and *APRIL* can support the growth of myeloma cells and that conversely an inhibitor of *BAFF* and *APRIL* can induce apoptosis of primary myeloma cells (108, 118). *BAFF* and *APRIL* genes are mainly expressed by the tumor environment, in particular monocytes and polynuclear cells (41). Of interest, we found a high production of *APRIL* in osteoclasts whereas bone marrow stromal cells did neither express *BAFF* nor *APRIL* (41). We also found that the gene coding for the *APRIL* receptor, *TACI*, had a clear-cut expression pattern in HMCL, being either "absent" or "present" (using the Affymetrix "call"). Respective data were confirmed by RT-PCR. Regarding primary myeloma cells, patients with myeloma cells highly expressing *TACI* (*TACI*++ myeloma cells) have a gene signature linked with microenvironment dependence in agreement with the production of these factors mainly by the tumor environment. Conversely, patients with myeloma cells weakly expressing *TACI* (*TACI*-/+ myeloma cells) have a plasmablastic gene signature. This was not found for other *BAFF* or *APRIL* receptors, *BCMA* or *BAFF-R*. Recent data on *APRIL* might help to understand the underlying mechanism. *APRIL*, unlike *BAFF*, is able to strongly bind to syndecan-1 (42). Of these two, only *APRIL* can bind at a high density to the surface of myeloma cells. This binding was abrogated by pretreatment of *APRIL* with heparin or of myeloma cells with heparitinase (unpublished observation). As *BAFF* does not

bind *TACI*, the property of *APRIL* to be concentrated at a high density on the myeloma cells membrane by syndecan-1 likely favors the emergence of myeloma cells that express the *APRIL* receptor *TACI*. This may explain the relevance of the microarray signature linked to the expression of *TACI*. Of note, we also found that some autonomous growing cell lines use *APRIL* as autocrine growth factor, in particular RPMI8226 (108). Thus, an interesting analogy exists between the results for *APRIL/TACI* and those for the *EGF* family. Both are important myeloma cells growth factors, in part due to the ability of syndecan-1 to concentrate them at a high density at the myeloma cells membrane. They are mainly produced by the bone marrow environment, but in some patients, myeloma cells can acquire the ability to express them and to become progressively independent of the tumor environment.

3.3.3. GEP and the interaction of myeloma cells with bone cells

DNA microarrays can also be used to gain insight in the interaction of myeloma cells with osteoblasts, osteoclasts and bone marrow stromal cells. Comparing the expression profile of myeloma cells from patients with or without osteolytic bone lesions, *DKK1* was found to be overexpressed in the first group (119). *DKK1* is an inhibitor of the *WNT* pathway and recombinant *DKK1* is able to block the differentiation of mesenchymal stem cells into osteoblasts. Of note, some *WNT* family members are also myeloma cells growth factors (120) and a high expression of *WNT5A* is often found in patients with MM and is a growth factor for myeloma cells (120, 121). Thus, an overexpression of *DKK1* in myeloma cells could possibly block the activation of the *WNT* pathway in myeloma cells and therefore the growth of myeloma cells. *DKK1*- myeloma cells have a plasmablastic phenotype (119). Using ATLAS macroarrays, HMCLs were found to overexpress the *FRZB/sFRP3* gene (28). *FRZB/FRP3* is a decoyed receptor for members of the *WNT* family blocking the activation of *WNT* receptors, and blocking also the differentiation of MSC into osteoblasts. As *FRZB/FRP3*

mRNA and protein are highly expressed by myeloma cells, *FRZB/FRP3* might block bone formation in MM. The *EGF* members could also be involved in a blockade of bone formation in MM. Indeed, amphiregulin and *HB-EGF* produced by myeloma cells and/or the tumor environment are able to block the differentiation of MSC into osteoblasts (122, 123). We also reported a high expression of genes which may contribute to the interactions of myeloma cells with bone cells: *GAS6, legumain and cystatin* C (16, 121). HMCLs expressed *TYRO3* and malignant and normal plasma cells expressed the two related receptors, *AXL* and *MERTK* (see Figure 5). *TYRO3* is a receptor with kinase activity, which is expressed in mature osteoclasts in the bone marrow. The ligand of these receptors is *GAS6* which is produced by normal and malignant PCs (Figure 5) (16).

4. Conclusion

Microarrays are a very powerful technology and give a precise overview of the known mechanisms involved in normal B-lymphocyte and plasma cell development and their physiological interaction with other cell populations, especially the bone marrow environment. The challenge will be to integrate to our knowledge of plasma cell biology the numerous genes that microarrays detect as induced or repressed during PC differentiation and that were never before described in this differentiation pathway. Similarly, microarrays will help to investigate the biology of plasma cell dyscrasias, the complex interactions between myeloma cells and the bone marrow environment and the major mechanisms of myelomagenesis underlying the overlapping distribution of a plethora of cytogenetic aberrations. Furthermore, it is likely that using advanced biostatistic approaches, a risk classification based on the expression profiling of the myeloma clone will provide a guide for a tailored treatment targeting myeloma cells and the tumor environment.

Legends to figures

Figure 1

The plasma cell transcription factor network. (A) Green boxes indicate transcription factors that are repressed during plasma cell differentiation and orange boxes transcription factors that are induced upon plasma cell differentiation. A solid line indicates a direct interaction of the transcription factor with the promoter of a target gene. Major events are the induction of *PRDM1/BLIMP1* secondary to the inhibition of *BCL6* activity, the repression of *PAX5* expression by *PRDM1*, that in turn drives the upregulation of *XBP1*. *XBP1* is mandatory for plasma cell differentiation and for the unfolded protein response. See text of gene abbreviation. (B) Histograms show the expression level of 10 transcription factors regulating B-lymphocyte and plasma cell differentiation. Gene expression is measured by pan genomic HG-U133AB Affymetrix oligonucleotides microarrays and the signal intensity for each gene is shown on the Y axis as arbitrary units determined by the GCOS 1.2 software (Affymetrix). Each histogram features the same 21 samples: 7 peripheral blood $CD27^+$ memory B-lymphocytes samples (green boxes), 7 $CD20^-$ $CD38^+$ plasmablasts samples obtained by in vitro differentiation of memory B-lymphocytes (orange boxes), and 7 $CD138^+$ bone marrow plasma cells samples (red boxes). If the gene is not detected, i.e. the gene has an "absent" detection call in the GCOS 1.2 software, the box is left white. Histograms were generated with our "Amazonia!" web site (http://amazonia.montp.inserm.fr/). Genes on the left are preferentially expressed in B memory samples whereas gene on the right are preferentially expressed in plasma cell samples. The level of significance of the expression level variation between the three sample classes was assessed using a non parametric Kruskal-Wallis test. NS : $P > 0.05$.

Figure 2

The plasma cell phenotype. Histograms show the expression level of 11 genes coding for cell surface proteins characterizing B-lymphocytes or plasma cells. See legend in Fig 1B.

Figure 3

Chemokine receptors. (A) Histograms show the expression of 5 chemokines receptors characterizing B-lymphocytes or plasma cells. See legend in Fig. 1B. (B) Schematic representation of the expression of *CCR7, CXCR5, CXCR4, CCR1 and CCR2* on memory B cells, plasmablasts and bone marrow plasma cells (BMPC).

Figure 4

Selectins and adhesion molecules. Histograms show the expression of 9 genes coding for adhesion molecules characterizing B-lymphocytes or plasma cells. See legend in Fig. 1B.

Figure 5

Growth factors and growth factor receptors. Histograms show the expression of 8 genes coding for receptors or receptor ligands characterizing B-lymphocytes or plasma cells. See legend in Fig. 1B. (*) Expression of *FRZB* in the same 21 normal B lymphocytes and plasma cells samples used in all histograms, plus CD138+ purified plasma cells from 19 patients with multiple myeloma (purple boxes). Note that the scale of the Y axis is different than in the histogram showing the expression of *FRZB* in nonmalignant B lymphocytes and PCs. Some MM samples display a striking overexpression of *FRZB* compared to *BMPC* samples.

Figure 6

Apoptosis and cell cycle. Histograms show the expression of 9 genes involved in cell cycle progression or apoptosis in B-lymphocytes or plasma cells. Note that *cyclin D1 (CCND1)* is

not expressed in normal B-lymphocytes or plasma cells (boxes are white), that *CCND2* is highly overexpressed in plasmablasts, whereas *CCND3* is expressed in all three cell categories with modest expression disparity across samples categories. See legend in Fig. 1B. ND : *P*-value not assessed because this gene was not detected in any sample.

Figure 7

Unfolded protein response (UPR). (A) A schematic view of the UPR in plasma cells in response to the expression of *XBP1* and immunoglobulins (Ig). With the increasing load of proteins in the endoplasmic reticulum (ER) due to the massive translation of Ig, the heat shock protein *HSPA5/BIP* is displaced from proteins such as *ATF6*, *ERN1/IRE1* and *EIF2AK3/PERK* to newly synthetized Ig. The dissociation of *HSPA5* from *ATF6* induces the cleavage of *ATF6*. The nuclear form of *ATF6 (ATF6(N))* induces the expression of target genes, including XBP1, by binding a ER stress response element (ERSE) in their promoter. The release of ERN1 from *HSPA5* induces the homodimerization of this kinase, self-phosphorylation and activation of its endoribonuclease activity. *ERN1* activity results in an unconventional splicing of *XBP1*: *XBP1(S)*. *XBP1(S)* is in turn able to activate the UPR by binding to the UPR response element (UPRE) in target genes. UPR genes includes genes involved in ER targeting, ER membrane translocation, protein folding, oxidative protein folding, misfolded protein degradation, protein glycosylation and ER-golgi trafficking. The release of *EIF2AK3* induces the activation of this kinase and the subsequent phosphorylation of *EIF2A*. Phosphorylation of *EIF2A* turns off protein translation and activates *ATF4*, which induces the expression of *CHOP* and *GADD34*. Overexpression of *CHOP* is associated induction of apoptosis. Orange boxes stand for genes that are found upregulated in plasma cells and purple boxes stand for genes whose expression does not change during normal plasma cell differentiation.

(B) Histograms show the expression of 22 genes coding for proteins involved in the UPR. See legend in Fig. 1B.

Figure 8

Expression of *Fibroblast growth factor receptor 3 (FGFR3)*, *Wolf-Hirschhorn syndrome candidate 1* (*WHSC1/MMSET)* and *cyclin D1 (CCND1)* in non malignant memory B lymphocytes (B) plasmablasts (PB) and bone marrow plasma cells (BMPC), in monoclonal gammapathy of unknown significance (MGUS), multiple myeloma (MM) and human myeloma cell lines (HMCL). Expression was measured by pan-genomic Affymetrix U133AB microarrays and visualized using our "Amazonia!" web site (http://amazonia.montp.inserm.fr/). See legend in Fig. 1B. The presence of t(4; 14) and t(11;14) translocations was evidenced by FISH analysis and is indicated on the histograms by colored circles : white circle : no t(4;14); red circle : t(4;14); grey circle : no t(11;14); green circle : t(11;14) in all myeloma cells; pale green circle : t(11;14) in a myeloma cell subclone only; no circle : no FISH analysis.

Figure 9

Syndecan-1 concentrates on the myeloma cell membrane large levels of *heparan-sulfate binding epidermal growth factor (HB-EGF)* family members that are produced by the tumor environment or by myeloma cells. This concentration step is critical for the activity of these growth factors on myeloma cells.

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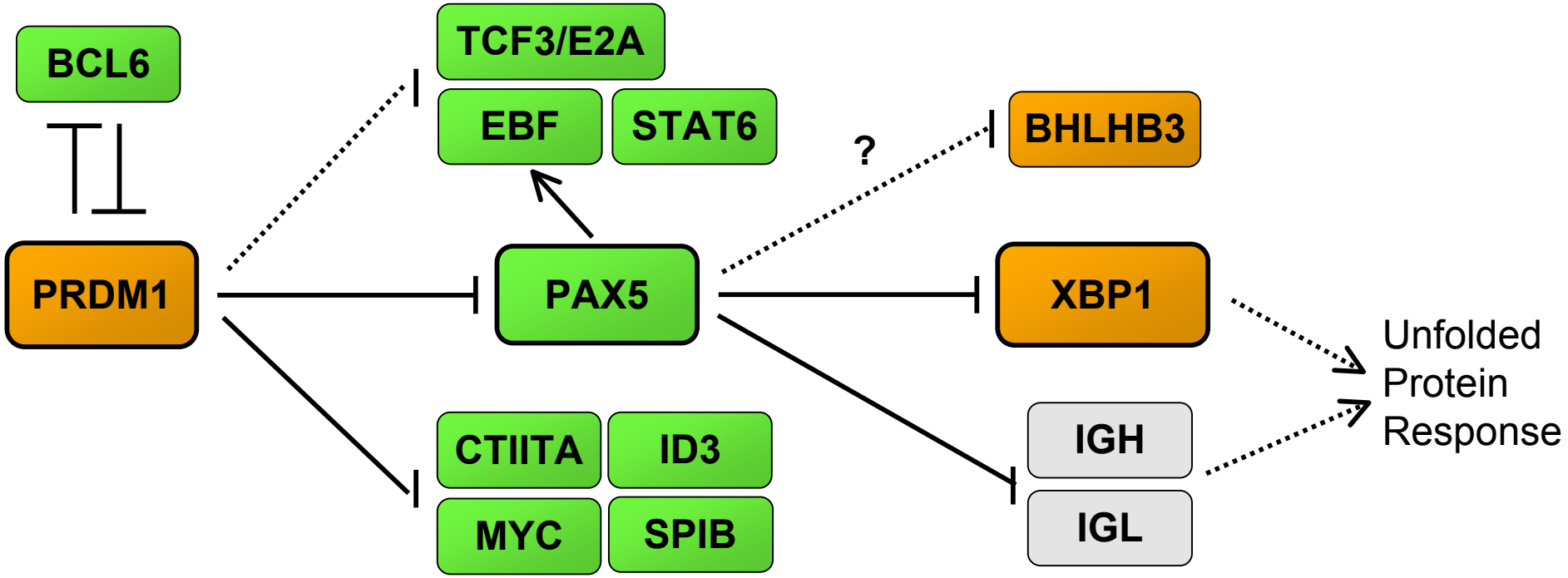
Figure 1A



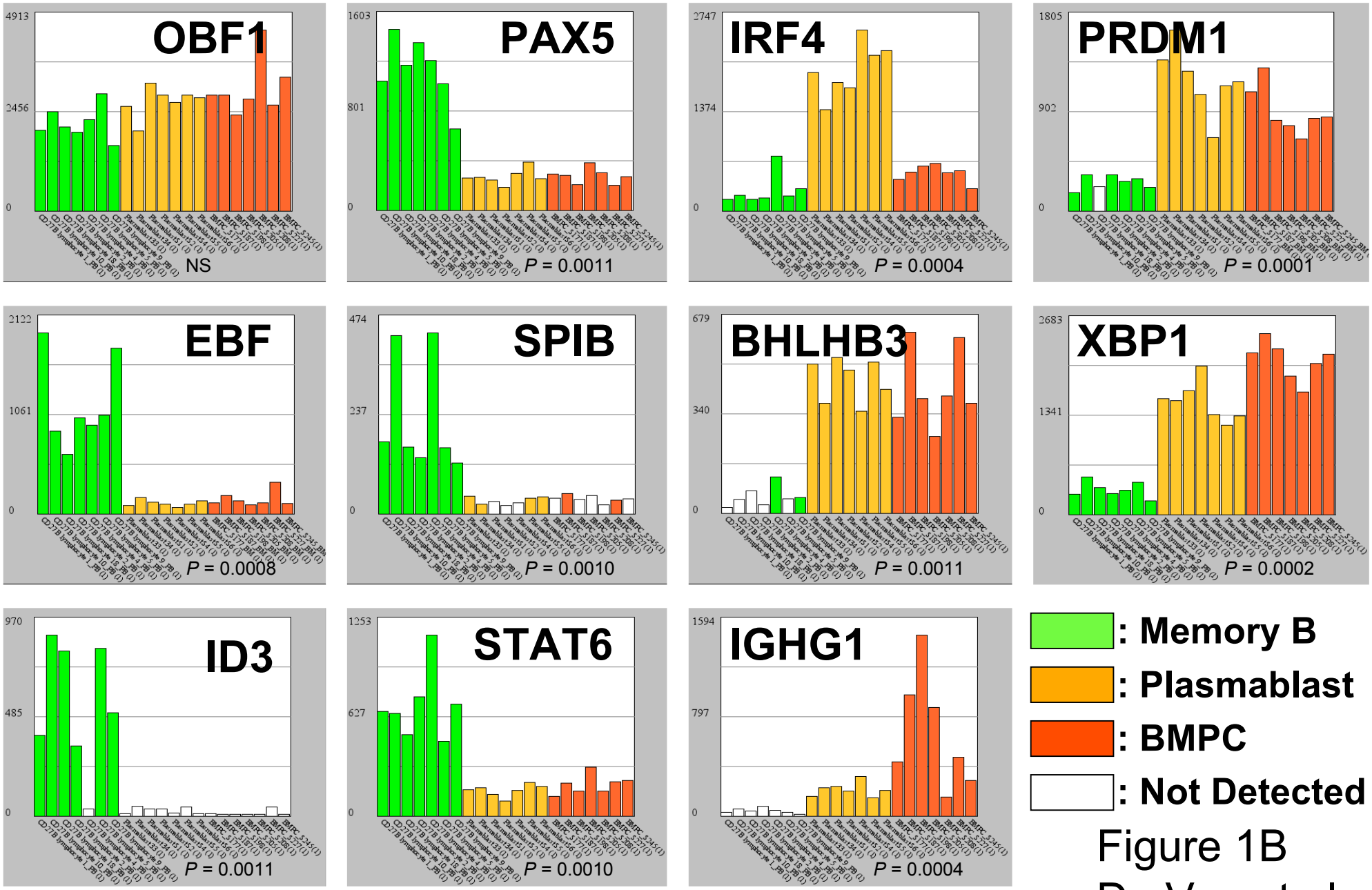
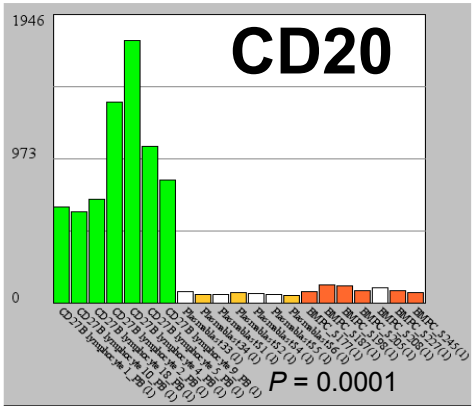
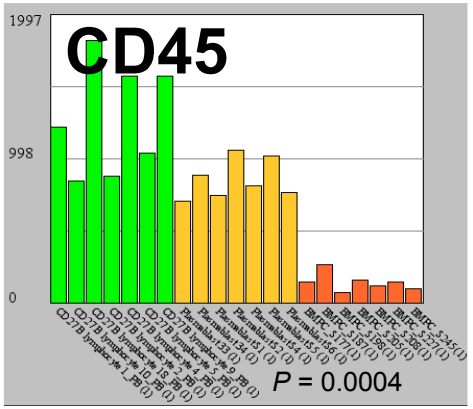
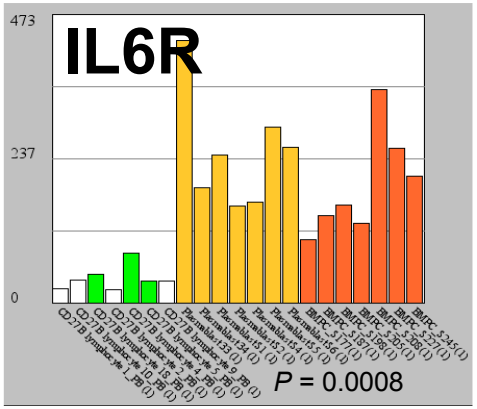
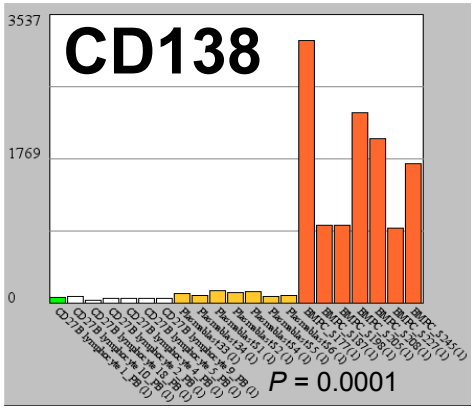


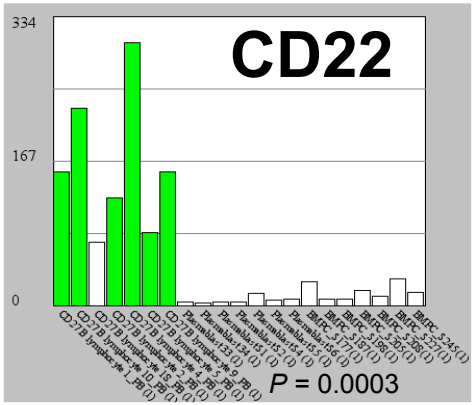
Figure 1B
De Vos et al.

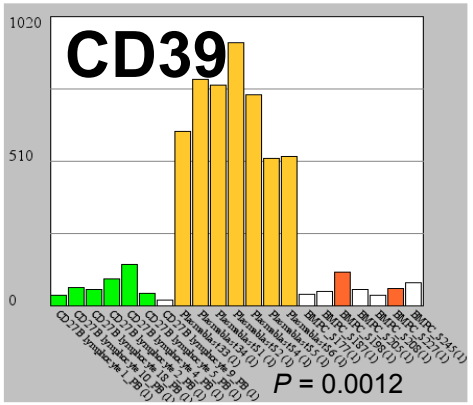


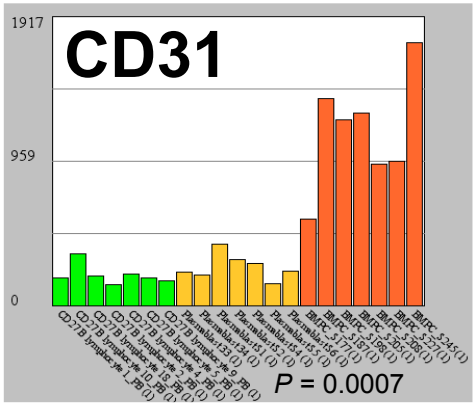


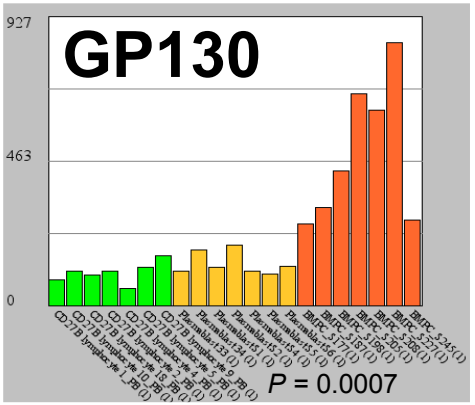


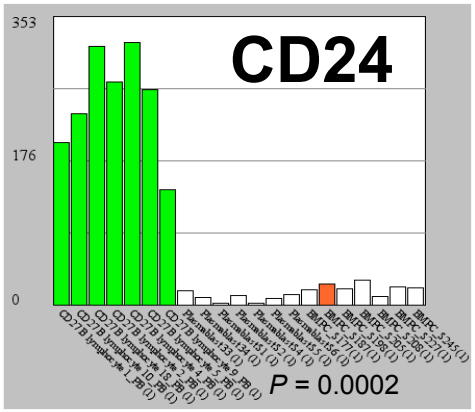


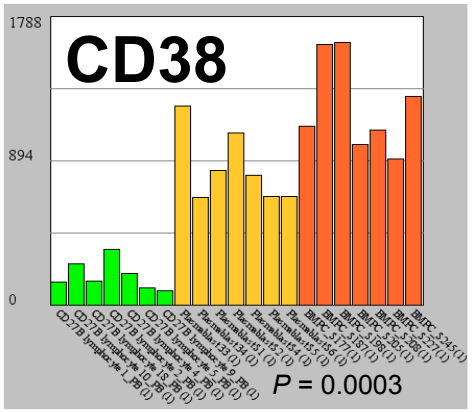


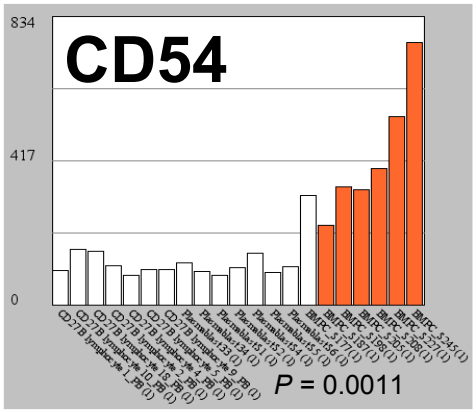












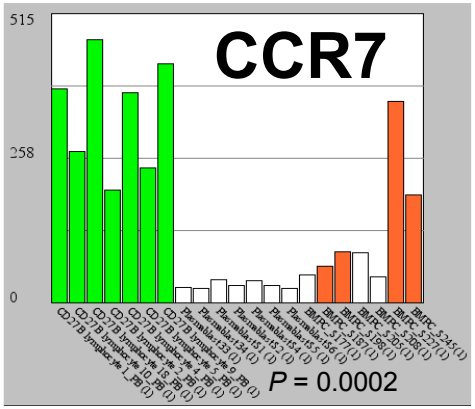
: Memory B

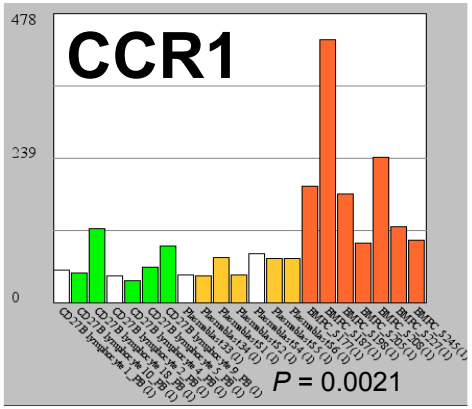
: Plasmablast

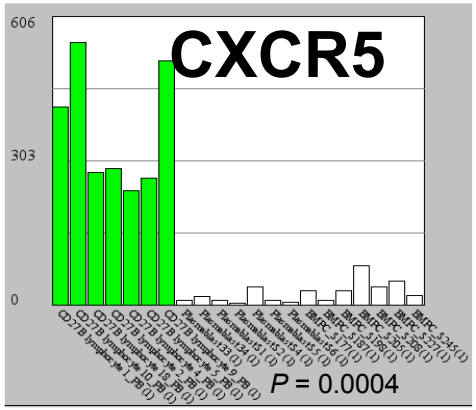
: BMPC

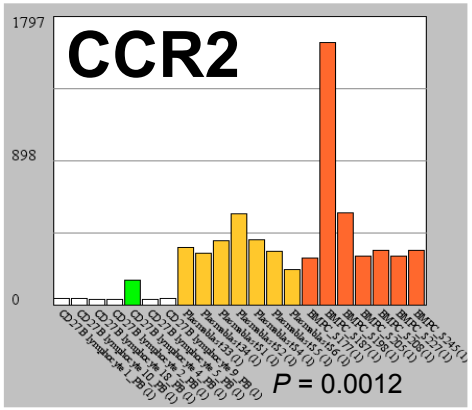
: Not Detected

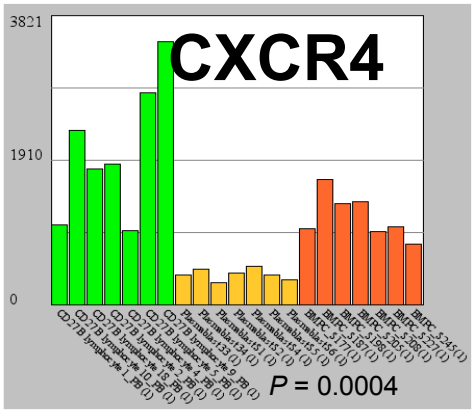
Figure 2
De Vos et al.











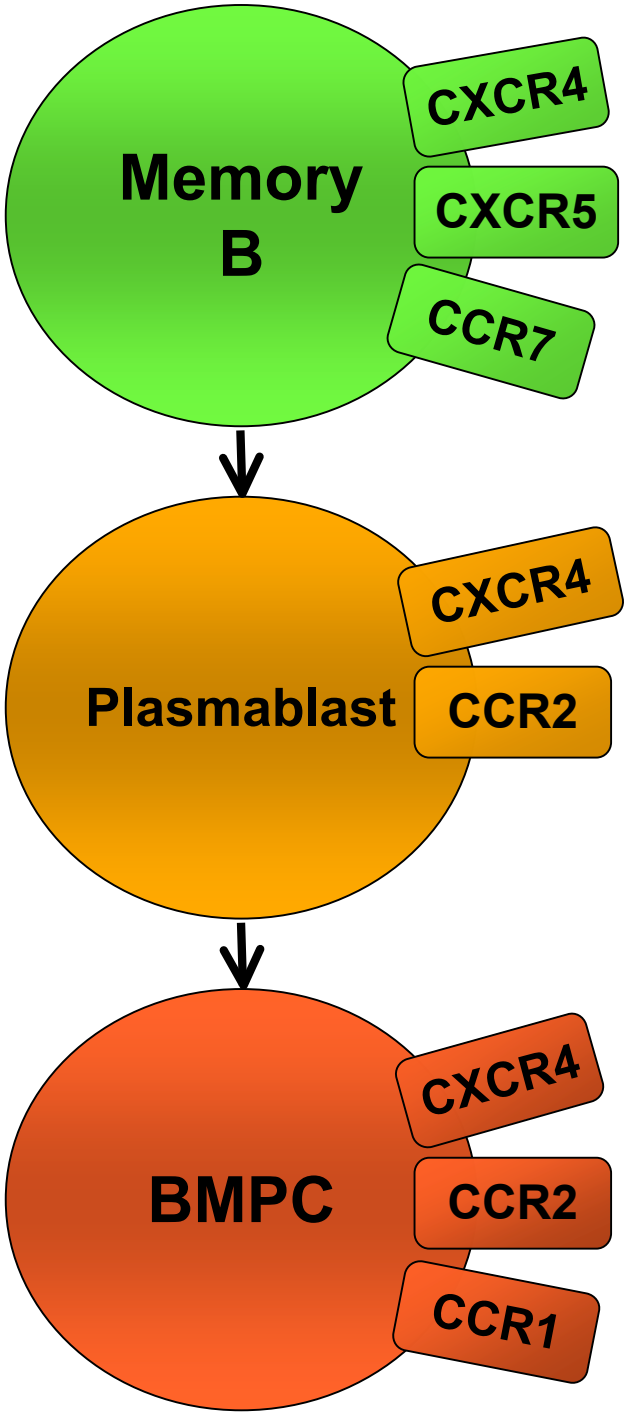
: Memory B

: Plasmablast

: BMPC

: Not Detected

Figure 3
De Vos et al.



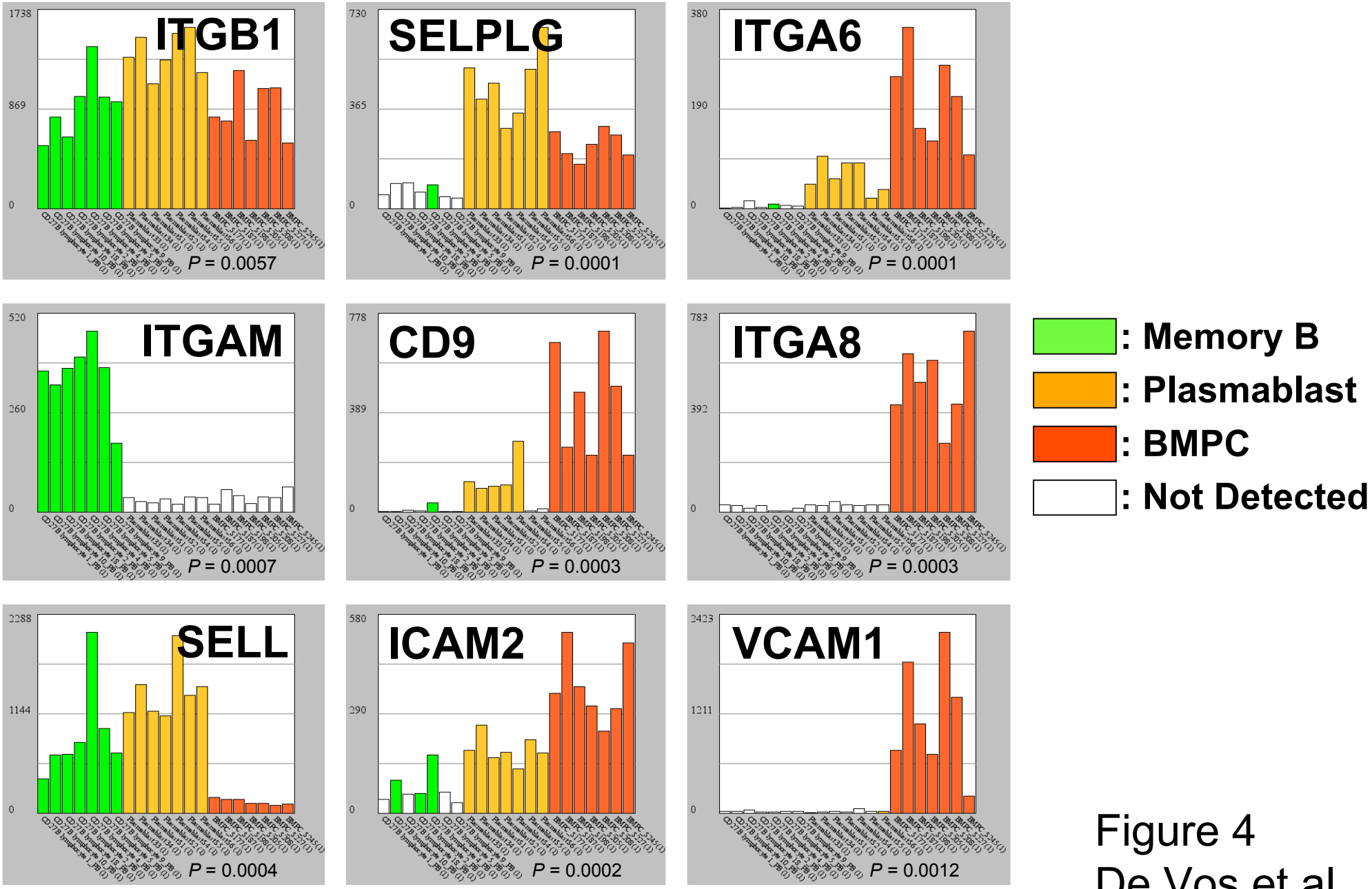
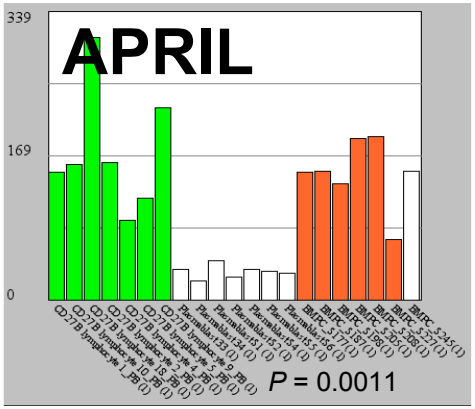
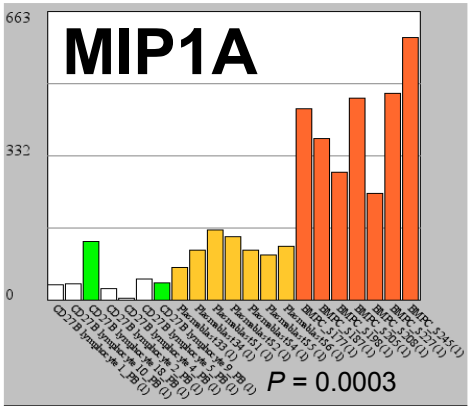
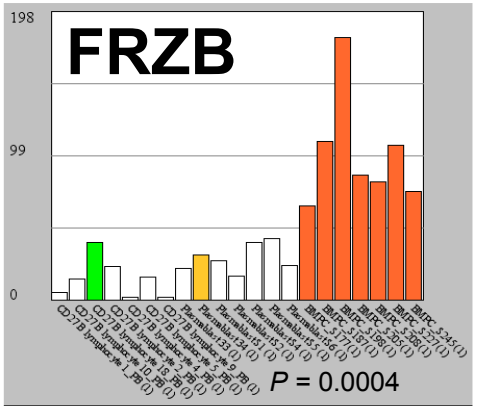


Figure 4
De Vos et al.







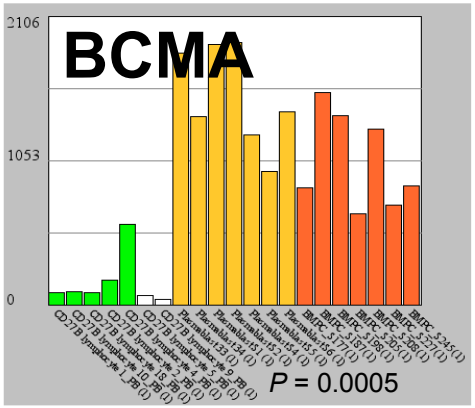
: Memory B

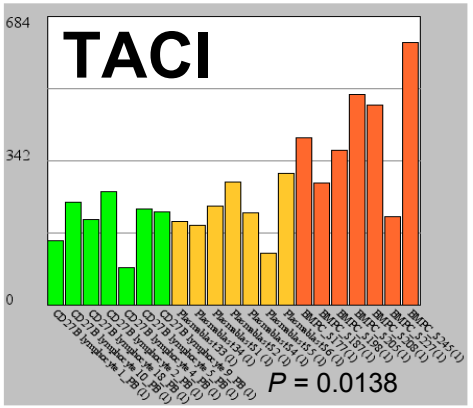
: Plasmablast

: BMPC

: MM

: Not Detected





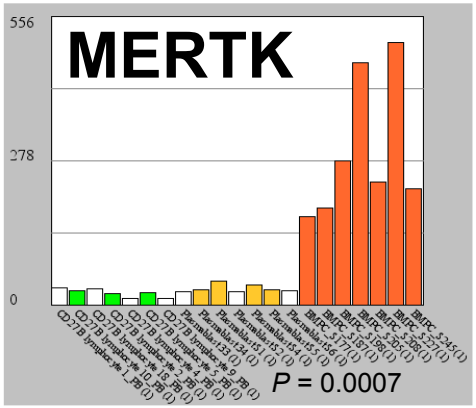
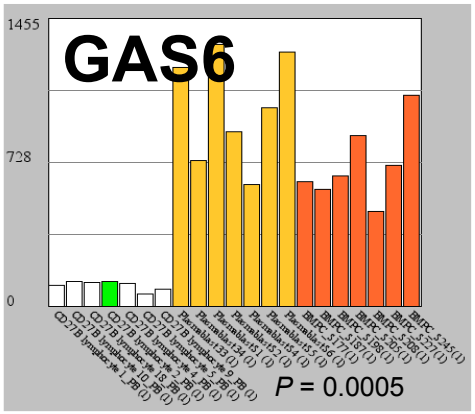
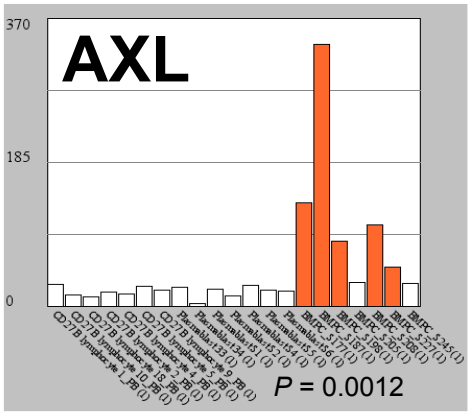
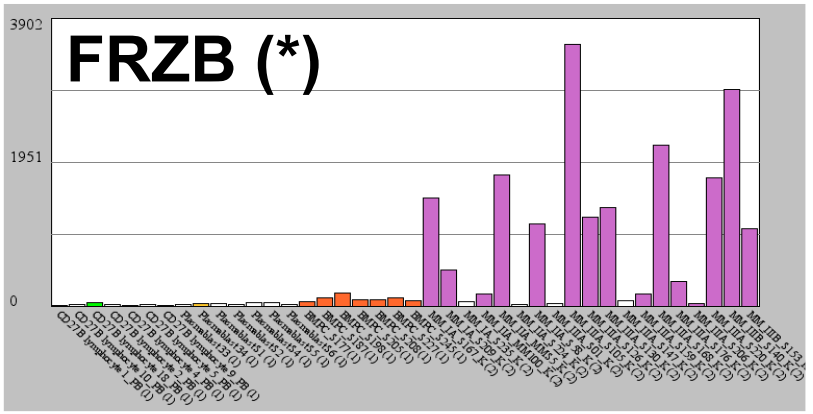
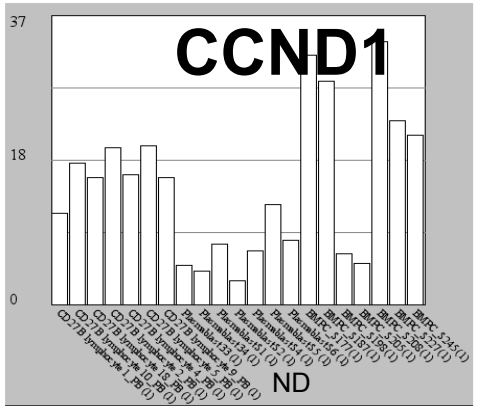


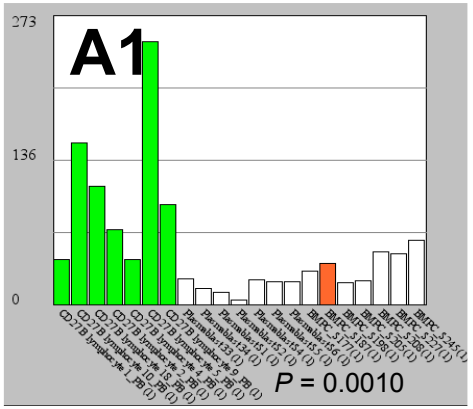
Figure 5
De Vos et al.

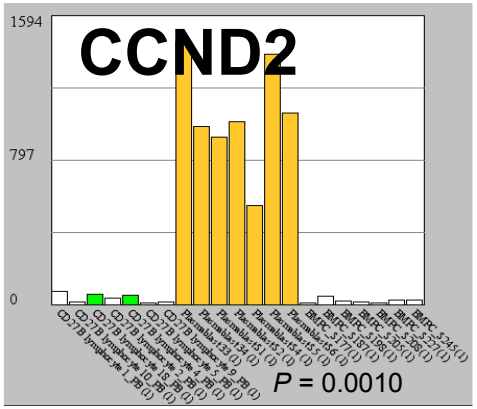


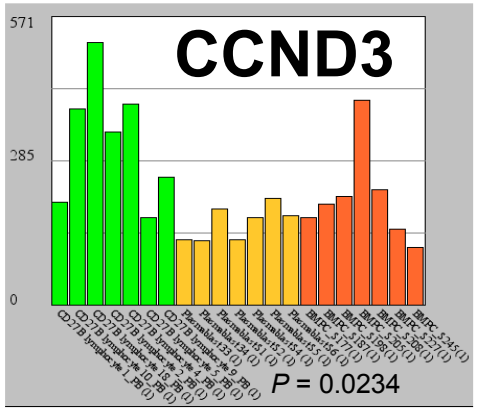


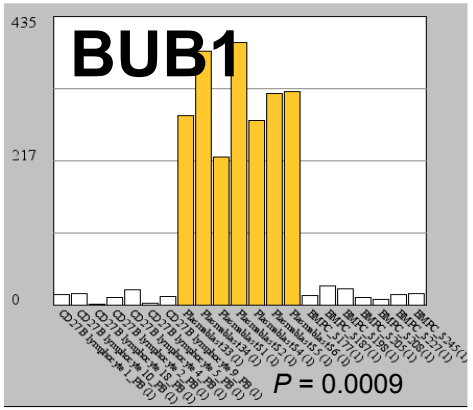


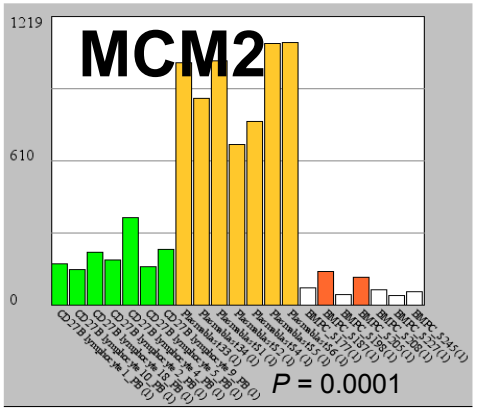


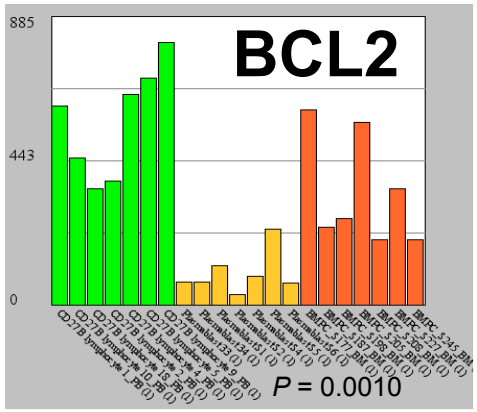


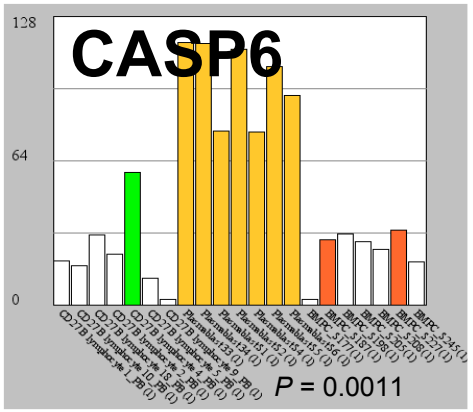


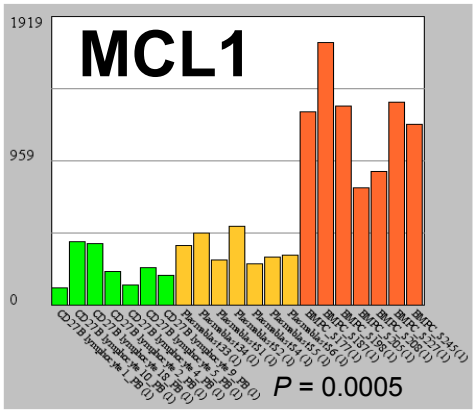












: Memory B
: Plasmablast
: BMPC
: Not Detected

Figure 6
De Vos et al.

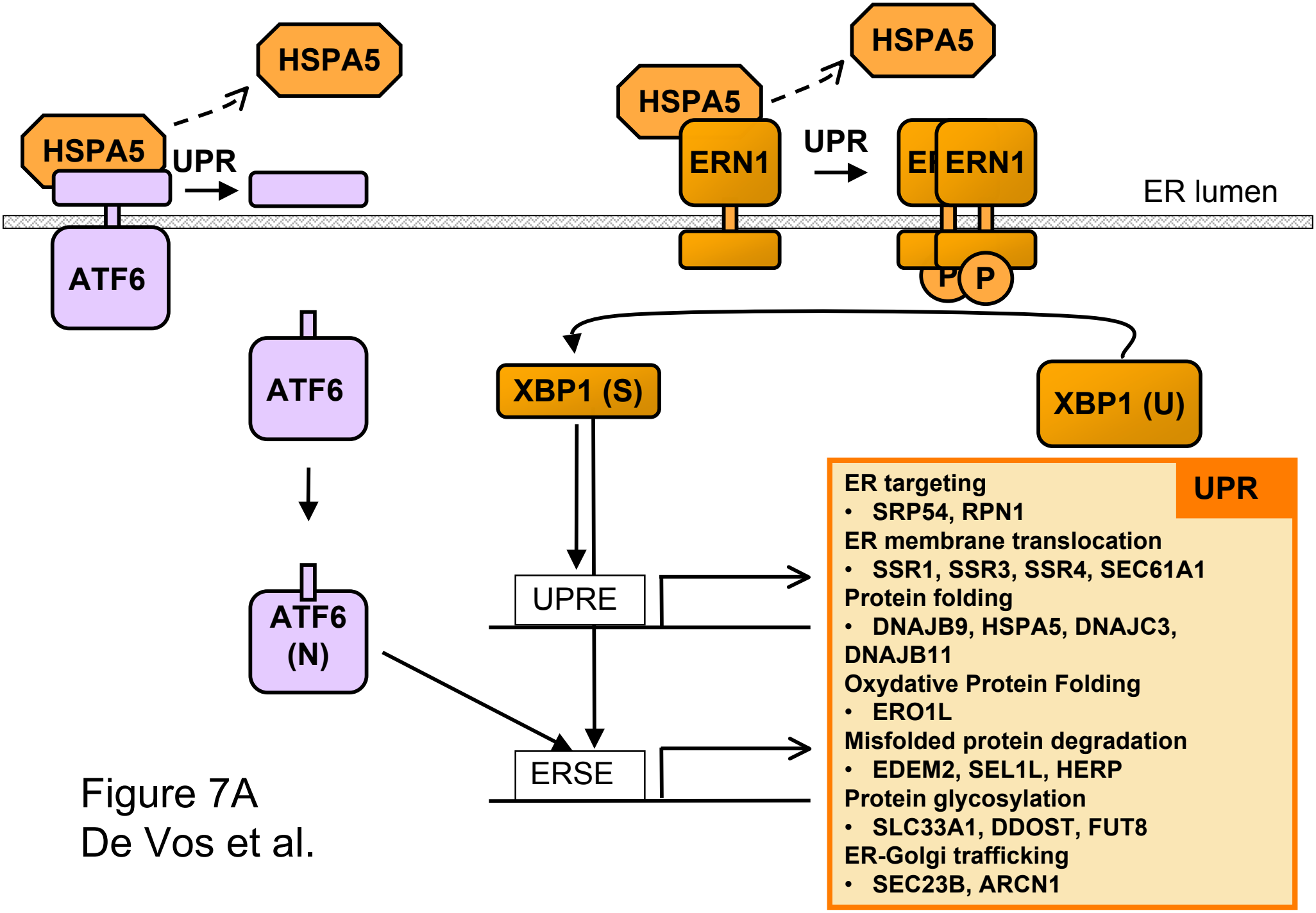


Figure 7A
De Vos et al.

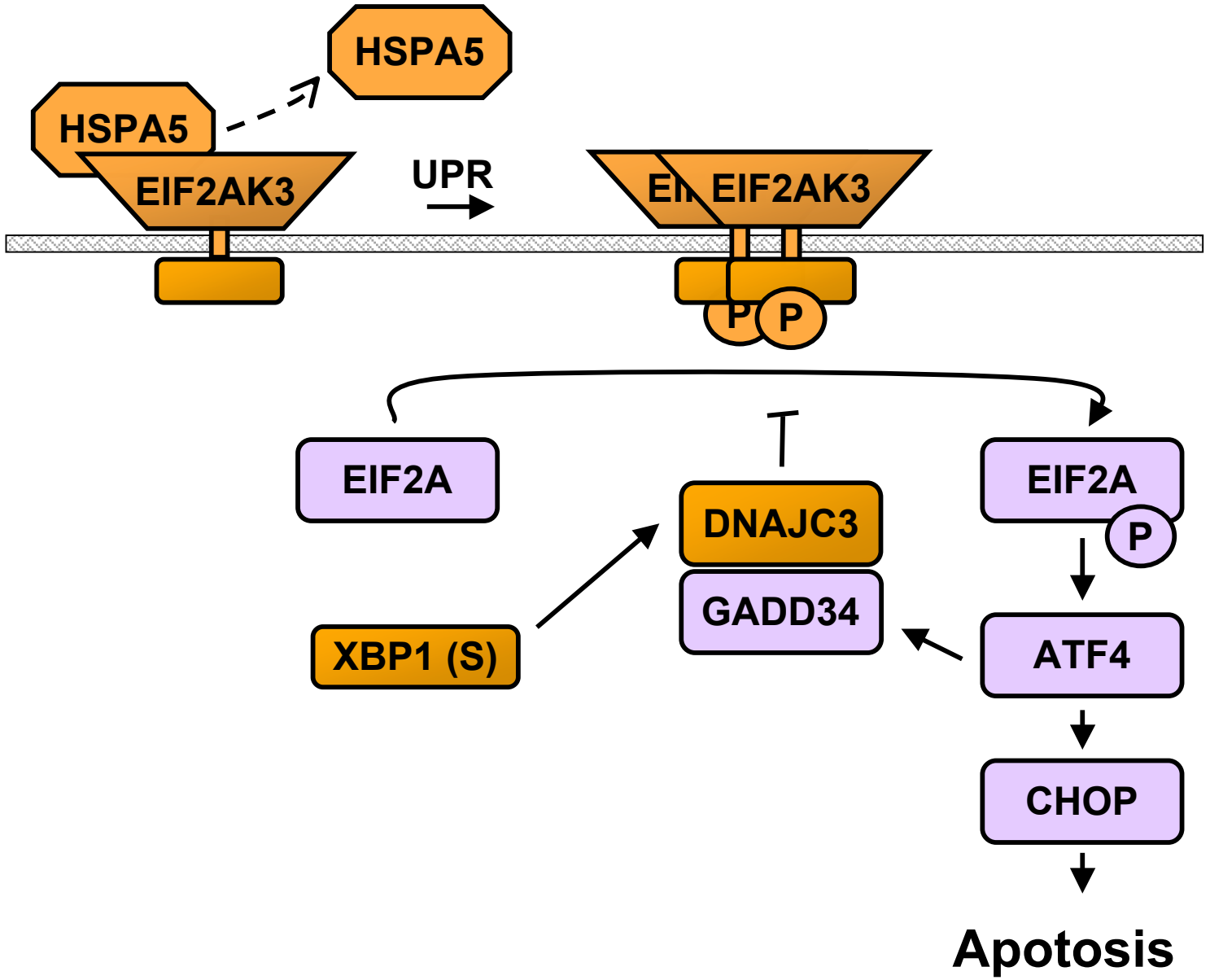


Figure 7A (continued)

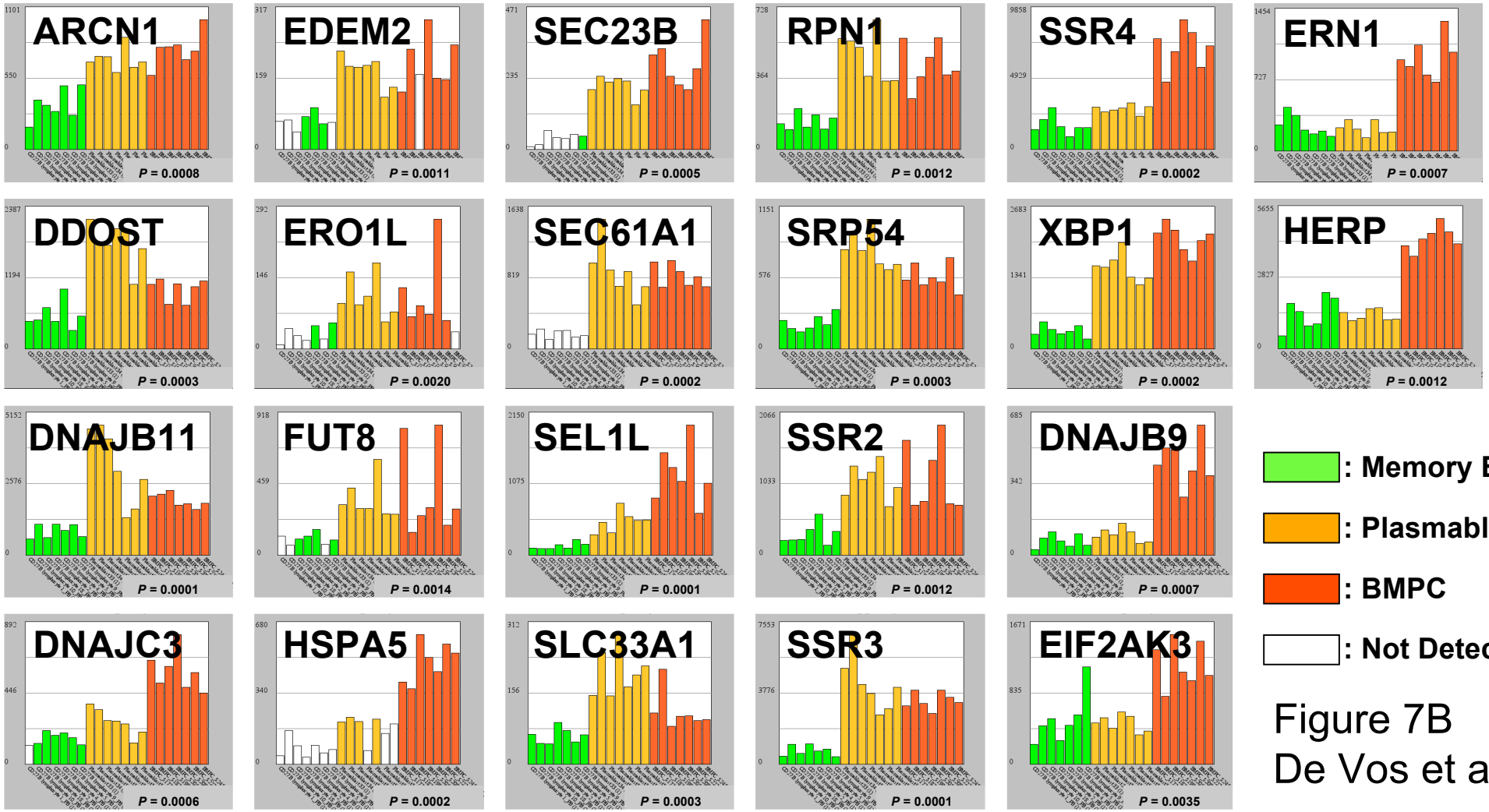


Figure 7B
De Vos et al.

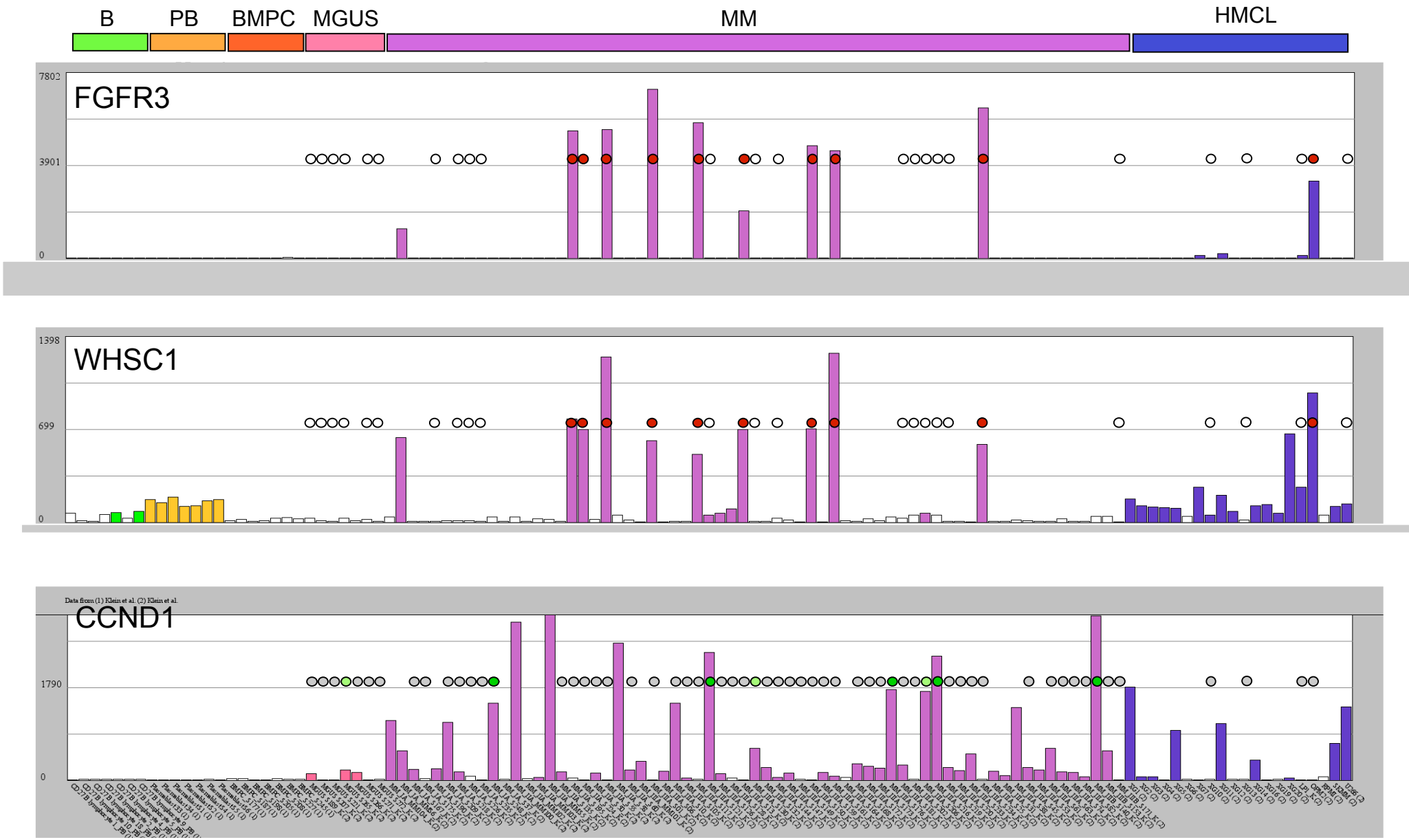


Figure 8 De Vos et al.

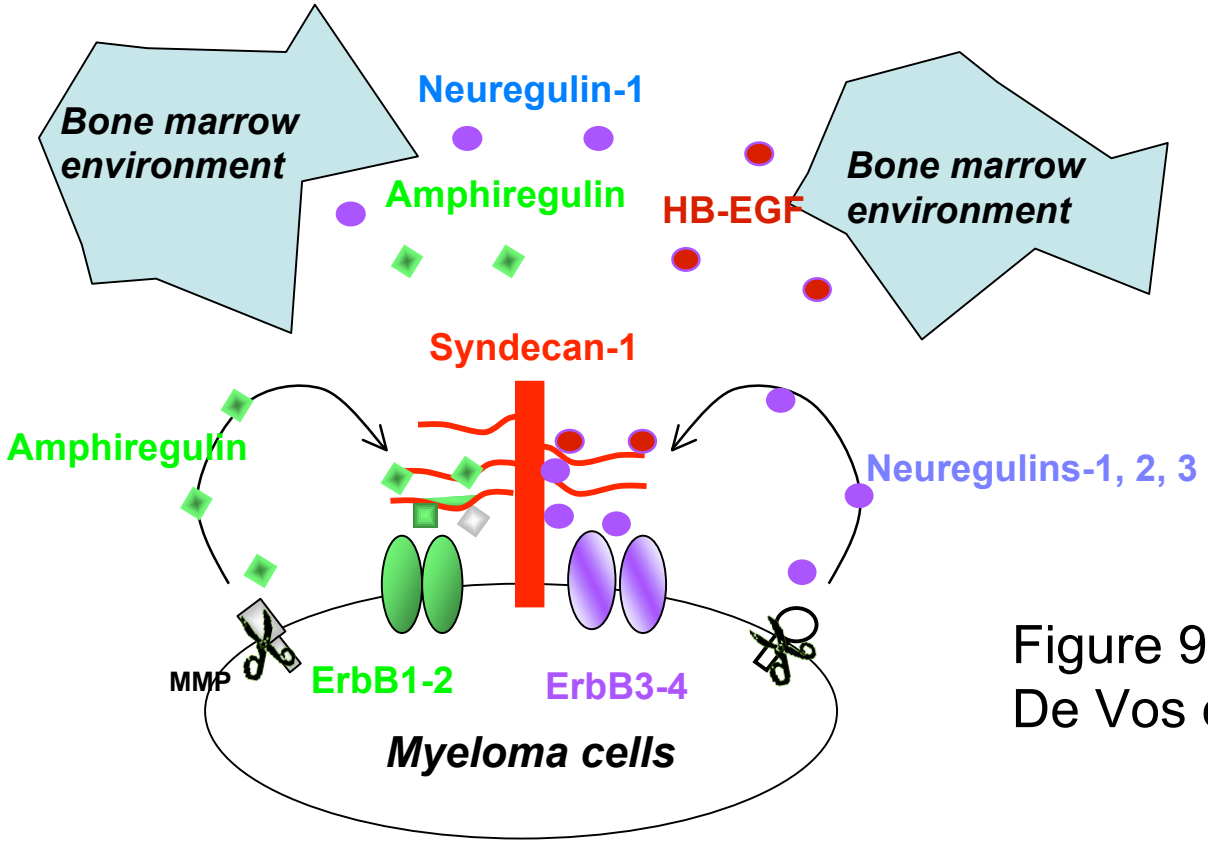


Figure 9
De Vos et al.